2 Methodology

2.1 Selection of model drugs and model LMWP for renal targeting.

Model drugs
Initially, NSAIDs, diuretics, antibiotics and cardiovasculars were considered as test drugs from the viewpoint of therapeutic interest for renal targeting (Fig. 7). Individual representatives were selected following the selection criteria summarized below:
a) Model compounds and model drugs were chosen on the basis of their coupling potential to proteins or spacer molecules as well as the potential to release the active compounds in the lysosomes. To this end, the model compounds and model drugs selected contained either a carboxyl group or a terminal amino group (for structures see supplement 6.1). It is important to emphasize that model compounds and model drugs lacking other functional groups that might interfere during the coupling reaction with the protein were preferred.
b) Knowledge of the pharmacokinetic properties of the compounds in animals.
c) The availability of selective and sensitive bioanalyses of parent drug and major metabolites.

Model LMWP
As model LMWP egg-white lysozyme was chosen, since:
a) its renal disposition has been extensively studied [138-140].
b) it contains six lysine residues, which are together with its terminal amino group potential sites for drug derivatization.
c) it is commercially available in a highly pure form.

2.2 Synthesis of drug-LMWP conjugates or drug-spacer candidates and selection of suitable drug-LMWP conjugates by *in vitro* methods.

Model bonds were chosen in relation to their expected cleavability in lysosomes and also their stability in plasma.

Cleavability of bonds in lysosomes and selection of model bonds
Lysosomes contain various hydrolytic enzymes (peptidases and esterases). In addition, the pH of lysosomes is 4-5, enabling acid catalyzed hydrolysis [106,109]. Therefore, with regard to parent drug regeneration both **enzymatic** and **chemical cleaving** potentials were evaluated.
Amide- and ester linkages were considered as model bonds. In principle these bonds are cleavable by lysosomal peptidases or esterases. In addition, an acid-sensitive *cis*-aconityl amide bond had been described for the conjugation of drugs with amino group [51]. This type of bond is cleavable at pH values less than 6 and may not require enzymatic cleavage. Various drug-protein conjugates or drug-spacer derivatives containing these model bonds were synthesized (supplement 6.1).
Selection criteria in the experiments *in vitro* were:
a) Stability of these conjugates in plasma.
b) Rapid and efficient drug regeneration in the renal lysosomal and/or cytosolic environment.

Design strategy for obtaining suitable drug-LMWPs

The working-hypothesis was that drug-spacer derivatives themselves can be considered as potential catabolites after protein digestion in the lysosomes. So, if a specific drug spacer molecule failed to be cleaved to render parent drug in the renal lysosomal and/or cytosolic environment, coupling of this derivative to the LMWP was regarded as useless, since it was expected that this would also result in (at least) incomplete drug release *in vivo*. Drug-spacer molecules which indeed rendered parent drug were used for further coupling to the protein and for evaluation of drug release from protein conjugates. If they failed to render parent drug, the primary spacers had to be optimized (length of spacer, degradability of the spacer). If these conjugates actually rendered parent drug, they were used for further testing *in vivo*.

Synthetical procedures

The syntheses of the compounds are described in supplement 6.1. The application of mini-scale synthesis was introduced for the synthesis of various drug-spacer derivatives. Using fractionated HPLC for purification in combination with (on and off-line) mass-spectrometry for identification, a relatively large number of compounds were synthesized, purified and characterized without performing elaborous multi-step syntheses. The validity of this procedure was verified by large-scale syntheses of some of these compounds (supplement 6.1).

Experiments *In Vitro*

In vitro testing was done by incubating the conjugates with renal cortex homogenates and lysosomal lysates. Cortex homogenates and lysosomal lysate incubations performed at pH 5 were used to mimic lysosomal digestion. Total cortex homogenate incubations were performed at pH 7.4 to include potential cytoplasmic digestion.

These homogenates and lysosomal lysates were prepared by the method of Maunsbach [141]. The validity of these preparations was verified using various substrates for carboxypeptidases and aminopeptidases and lysosomal marker enzymes, like acid phosphatase. Both cortex homogenates and lysosomal lysates were used to limit possible shortcomings of these individual preparations due to purification artifacts or autolysis of specific enzymes.

To obtain additional information on the renal handling of drug-LMWP conjugates also at the cellular level, other *in vitro* techniques may be of use in the future. These techniques include the isolated perfused kidney, the isolated perfused tubule, kidney slices, and proximal tubular cells in culture [72]. An advantage of these techniques is that they may enable to study the fate of the conjugated drug and carrier protein (i.c. uptake and endotubular drug release) in a relative simple setup in intact cells and may "bridge" the present *in vitro* and *in vivo* methods. The major disadvantage of some of these techniques is the short maintenance period of viable tissue, especially when measurements over more than 4 hours are required. For instance, the isolated perfused kidney does not allow reliable measurements over a larger period than 2 h. In this regard it should be noted that endorenal drug release may require several hours (Chapter 3). Also down-regulation of transport receptors in cultured cells may occur [72].

2.3 Testing of suitable drug-LMWP conjugates by *in vivo* methods.

Experiments *in vivo*

In vivo methods were designed such that comparative pharmacokinetic and (if possible) also pharmacodynamic studies could be performed with drug-LMWP (1:1) conjugates. As controls equivalent doses of mixtures of the drug and the LMWP (i.e. lysozyme) were administered to freely moving animals.

In these experiments, heart cannulated rats [142] were placed in metabolic cages. To obtain and maintain sufficient urine production for urine collection, the animals were infused with 5% glucose up to 3 $ml.h^{-1}$. The metabolism cages were further equipped with infrared urine detectors, fraction collectors and recorders for automated and timed urine sampling. The experimental setting allowed simultaneous blood and frequent urine sampling without disturbing the animals.

A major advantage of this technique is its potential to study drug disposition and excretion over a long period of time (up to 5 days). In addition, the same animals can be used in the control experiments, thereby avoiding the problem of interindividual variation and also to reduce the total number of animals in the particular experiments.

Analysis of drugs and protein

Drugs and/or (major) metabolites c.q. catabolites were assayed in plasma and urine by HPLC methods. Specific and sensitive HPLC methods were developed using fluorimetric detection and electrochemical detection. Unfortunately, sufficient quantities of radiolabeled drugs were not available. Measurement of radioactivity would have been convenient for establishing total drug concentrations in the kidneys.

The proteins were analyzed by enzymatic assays or by radioiodination using iodine-125 or iodine-123. In addition, the fate of ^{123}I-labelled proteins was followed non-invasively by gamma-camera imaging of anesthetized rats.